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Design and expression of a chimeric vaccine candidate for avian necrotic enteritis

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Abstract

Necrotic enteritis is an economically important disease of poultry mainly caused by *Clostridium perfringens*. The bacteria release multiple toxins of which NetB, alpha toxin and TpeL have been reported to play important roles in pathogenicity and/or severity of the disease. In this study, the sequence of clostridial toxins NetB, alpha toxin and TpeL were analyzed using bioinformatics tools to determine protein domains with high immunogenicity factor. Several chimeric trivalent proteins consisting of the immunogenic regions of the three toxins were designed and evaluated. The separate regions were fused together using rigid linkers. Based on a modeled tertiary structure, a proper combination was selected and expressed in a bacterial host (*Escherichia coli*) and successfully purified. The expression of the chimeric protein was further verified by western blotting. The ability of the immunized serum in recognizing each individual subunit of the chimeric protein was also examined. Circular dichroism was used to evaluate the predicted secondary structure of the chimeric protein. *In vitro* potency test demonstrated that the serum from a rabbit immunized with the chimeric protein is able to partially neutralize Alpha toxin, hence the construct can potentially be used as a vaccine against *C. perfringens*.

Key words: Alpha toxin, chimeric protein, necrotic enteritis, NetB, TpeL

Introduction

Avian necrotic enteritis (NE) is a common and severe gastrointestinal disease in broiler flocks causing up to 40% mortality if untreated (McDevitt *et al.*, 2006; Keyburn *et al.*, 2008) and leading to a significant financial loss for poultry producers (Dahiya *et al.* 2006; Lovland and Kaldhusdal, 2001). NE is characterized by lesions in the mucosa of the small intestine (Kaldhusdal and Hofshagen, 1992) resulting in severe and distinct pathological symptoms. The disease is also found in a less severe sub-clinical form that is not fatal but causes reduced digestion, weight gain and growth (Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 2001; Shojadoost *et al.*, 2012). It is believed that the Gram-positive rod-shape bacterium, *Clostridium perfringens* type A, is the main cause of NE (Titball *et al.*, 1999; Keyburn *et al.*, 2008). General worldwide tendency toward the reduction of antibiotic usage in food

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animals, including broilers, has resulted in the elevation of this fatal disease (Van Immerseel *et al.,* 2009) and further underscores the need for an effective vaccine against *C. perfringens*.

Many different agents are proposed to be important in the process of the disease development and progression, especially toxins secreted by *C. perfringens*. One of these toxins, alpha toxin is shown to be important in NE development or at least play a role in pathogenesis of the disease according to several studies (Lovland *et al.*, 2004; Cooper *et al.*, 2009). This bi-functional phospholipase C (PLC, lecithinase) and sphingomyelinase enzyme consists of two separate domains: the N-terminal domain with catalytic activity and the C-terminal domain that involves in binding of the enzyme to the cell membrane. The toxin does not have hemolytic activity in the absence of the binding domain (Schoepe *et al.*, 2001; Nagahama *et al.*, 2002; Uppalapati *et al.*, 2013).

Furthermore, a novel toxin, necrotic enteritis toxin, B-like (NetB), with strong association to NE has been identified (Keyburn *et al.*, 2008). NetB is a pore-forming toxin that belongs to the β-pore-forming toxin family (Savva *et al.*, 2013). It is shown that that there is a direct correlation between NetB toxin production by *C. perfringens* and manifestation of NE symptoms in chickens infected with the bacteria (Keyburn *et al.*, 2008). This implies the important role of this toxin in the pathogenesis of the disease (Keyburn *et al.*, 2010). It is also reported that no gut lesions were developed by NetB mutant *C. perfringens* in experimentally infected poultry (Keyburn *et al.*, 2008). However, vaccination with NetB results in partial protection against NE (Jang *et al.*, 2012; Fernandes da Costa *et al.*, 2013; Keyburn *et al.*, 2013a, 2013b).

Another clostridial toxin, TpeL, can induce apoptosis and also claimed to be effective in increasing the severity of the NE in poultry, as TpeL-positive strains of *C. perfringens* cause more fatal and more progressive disease (Coursodon *et al.*, 2012; Guttenberg *et al.*, 2012). It is shown that the N- and C-terminal regions of TpeL are important in its cytotoxicity while the C-terminal region plays a role in binding of the toxin to the cells (Nagahama *et al.*, 2011).

NE is considered to be a multi-factorial disease where many agents (toxins) seem to play a role in the etiology of the disease (McDevitt *et al.*, 2006; Van Immerseel *et al.*, 2009; Timbermont *et al.*, 2011). Thus, an effective vaccine should be able to at least provide protection against the main toxins produced by *C. perfringens*. Recently, two separate studies reported that co-administration of recombinant NetB and the C-terminal of alpha toxin can make protection against NE (Jiang *et al.*, 2015; Fernandes da Costa *et al.*, 2016).

In the present study, a chimeric protein was designed based on epitopes of the three different *C. perfringens* toxins, NetB, alpha toxin and TpeL. The constructed chimeric protein was cloned and expressed in a bacterial host and successfully purified. The protein was used in immunization of rabbits and the produced sera examined proved to contain antibody against the chimeric protein. It is also shown that the sera can recognize each recombinant toxin.

Materials and methods

B-cell epitopes prediction and sequence analysis

Amino-acid sequences of three toxins 'NetB' (accession number ACN73256.1), 'Alpha toxin' (accession number CAA35186.1) and 'TpeL' (accession number EDT23010.1) were obtained from GenBank. To determine their antigenicity, toxin protein sequences were analyzed separately using VAXIJEN software (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). The potential epitopic regions in the tertiary structure of the proteins were predicted using BCEPRED (http://www.imtech.res.in/raghava/bcepred/bcepred_submission.html). EXPASY was used for identification of the functional the domains the toxin proteins.

Construct design

Different regions of the toxin proteins were selected for making the chimeric fusion protein based on parameters including Ag-index, epitope region, hydrophobicity and function. The selected protein domains consisted of amino acids 150–322 from mature NetB, amino acids 256–370 from signal peptide truncated alpha toxin) and the final 120 amino acids from C-terminal of TpeL. Each of these selected protein domains was again submitted to VAXIJEN to check whether the sequences are still predicted to be antigen by the software. Four different combinations of the three selected protein fragments were designed. The different arrangements were as follow: 'NetB–Alpha toxin–TpeL' or NAT, 'NetB–TpeL–Alpha toxin' or NTA, 'Alpha toxin–NetB–TpeL' or ANT and 'Alpha toxin–TpeL–NetB' or ATN. The fragments were fused together using two identical rigid hydrophobic linker A(EAAAK)4A. Due to the high hydrophobicity and of the TpeL, the construct was preferred not to be started with TpeL. A sequence of six histidine (His tag) was also added to the end of the construct for further purification of the recombinant protein with nickel beads.

Protein structure analysis

GOR IV secondary structure prediction method was used for prediction of the chimeric protein's secondary structures (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html). Predict Protein server was then used for further sequence analysis such as identifying low-complexity regions, regions with no regular structure, transmembrane helices, solvent accessibility, coiled-coil regions, disulfide bonds, subcellular localization and functional annotations (https://www.predictprotein.org/).

To predict the tertiary structure of the hypothetical chimeric protein, all four different combinations were analyzed using I-TASSER program (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The combination with best tertiary structure in which all antigenic areas were exposed was selected as the main candidate. Further validation was carried out by uploading protein 3D structure to the ProSA web server (Wiederstein and Sippl 2007).

Gene optimization

The selected hypothetical chimeric protein sequence was back translated to DNA (http://www.bioinformatics.org/sms2/rev_trans.html) and optimized for expression in *Escherichia coli* using gene optimization software (https://www.dna20.com/resources/genedesigner). The mfold program (http://mfold.rna.albany.edu/?q = mfold) was used for evaluation of the RNA secondary structure of the designed chimeric gene, before and after codon optimization.

Expression of the chimeric protein

The chimeric gene was synthesized at Shine gene Molecular Biotech, Inc. (Shanghai, China). The recombinant pUC57 plasmid harboring the designed construct was transformed into the competent *E. coli* DH5α for amplification. Ampicillin selection (100μg/ml) and some control tests ensured the presence of the recombinant plasmid in the bacteria. After extraction, pUC57 and pET-26b (+) were digested by NdeI and XhoI restriction enzymes. Fragments were purified

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from gel and then inserted into the expression vector pET-26b (+) through enzyme digestion and ligation reactions. The resulting plasmid was transformed into competent host *E. coli* BL21(DE3). The transformed bacteria were cultured in LB medium at 37°C supplemented with kanamycin (50 µg/ml). After the OD of bacteria culture reached 0.6–0.7, the bacteria was induced by isopropylthio-β-D-galactoside (IPTG) at a final concentration of 1 mmol/l and then incubated for 5 h at 30°C. The induced bacteria were collected by centrifugation and the chimeric protein was purified.

Protein purification

The His-tagged chimeric protein(s) was purified by Ni-NTA Magnetic Agarose Beads (QIAGEN Inc., USA). After centrifugation, the pellet was suspended in lysis buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 8.0) and was shaken at room temperature for 1 h. Then, the cell lysate was centrifuged at 10 000*g* for 30 min, the cleared supernatant was added to Ni-NTA resins. After 2 h of incubation at room temperature, resins were washed five times with washing buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05%, imidazole 20 mM). At last, the chimeric protein was eluted by adding elution buffer containing (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05%, imidazole 250 mM). The chimeric protein was then dialyzed against decreasing gradient of urea concentrations in order to eliminate urea from the solvent. Protein samples were analyzed by SDS-PAGE and western blotting.

Circular dichroism analysis

In order to validate the predicted secondary structures of the heterologous protein, circular dichroism (CD) analysis was performed. CD data were collected using JASCO J-810 CD spectrometer (Tokyo, Japan). The experimental setup measures the ellipticity, $[\theta]$, (deg cm^2/dmol), of the electromagnetic wave that traversed the sample. The molar ellipticity, $[\theta]$, at each wavelength was determined by the following equation:

$$[\theta] = (\theta \times 113)/cl,$$

where c is the protein concentration in the solution (mg/ml), l is the length of the light beam's path and θ is the measured ellipticity in degrees in the far-UV region. The amount of secondary structure of the chimera was calculated by J-800 Protein Secondary Structure Estimation Program (Model JWSSE-480) for Windows, and according to the method explained previously by us (Bathaie *et al.*, 2003, 2011). The concentration of 0·25 mg/ml chimera in phosphate-buffered saline buffer was used to investigate the secondary structures (far-UV region) of the chimera. The CD spectrum of the cuvette containing dialysis buffer was recorded and subtracted from the same solution containing the chimeric protein.

Separation and purification of chimeric protein subunits

For obtaining each subunit, *netB*, alpha toxin and *tpeL* were amplified individually by PCR, expressed in *E. coli* and purified. Three sets of primers that were used for isolation of subunits are listed in Table I.

Western blot

Western blotting was done against the complete chimeric protein as well as each of its individual subunits. The chimeric protein and each of its purified individual subunits were separated on SDS-polyacrylamide gels and transferred onto PVDF membrane (Roche, Germany). Further analysis was done according to the manufacturer instruction. Anti-rabbit HRP was purchased from GE Healthcare Life Sciences.

Serum neutralization test

According to the pharmacopeia 2015, titration of standard toxin and standard 10 IU/ml anti-toxin was used for measuring the amount of antibody in the rabbit pool sera (RPS). A group of 10 Dutch rabbits were injected with 200 µg/ml of purified chimeric protein (subcutaneous). Three weeks later, second injection at the same dose was given. Two weeks later, the blood samples were collected and the sera were pooled (RPS). The units of *C. perfringens* type A alpha antitoxins in RPS were estimated by titration in the first group of mice with the weight of 18–20 g. Based on their mortality, units of alpha antitoxins were calculated. In the second group, each mouse was injected with 0.5 ml of normal saline as a negative control.

Results

Chimeric gene construction

Epitopes of three *C. perfringens*'s toxins, NetB, TpeL and alpha toxin were selected for construction of chimeric gene expression cassettes. For giving flexibility and proper separation of functional domains, the selected regions of the three toxins were linked together using two A(EAAAK)4A linkers. This linker consists of four EAAAK repeats, which were expected to form a hydrophobic α-helix in the chimeric protein. Different combinations of the three toxins were designed. The sequential arrangement of the fragments and linkers for one of the combinations is depicted in Figure 1.

The amino-acid sequence was back translated and codon optimized for expression in *E. coli*. The chimeric protein was further analyzed for its GC content and sequence complexity. Codon adaptation index for optimized chimeric gene was determined to be 0.67. The GC content of the gene was 44% after optimization. Restriction enzyme sites including NdeI and XhoI were introduced at two ends

Table I. List of oligonucleotides

Name	Sequence	Restriction site
NBF1 (forward primer for *netB*)	TATACATATG ATTGGTTATTCTATTGG	NdeI
NBR1 (reverse primer for *netB*)	TCATCTCGAG CAGGTAATATTCGATTTTGTG	XhoI
ATF2 (forward primer for alpha toxin)	TATACATATGGAACTGGTCGCGTACATC	NdeI
ATR2 (reverse primer for alpha toxin)	TCATCTCGAGTTTGATATTGTAGGTAGAGTTAC	XhoI
TPF3 (forward primer for *tpeL*)	TATACATATGTATTCTTTCATCAACGATATTATC	NdeI
TPR3 (reverse primer for *tpeL*)	TCATCTCGAGGTCACGGTAACCGATGATC	XhoI

Underlined letters indicate restriction sites for NdeI and XhoI, respectively.

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Fig. 1 Schematic representation of 'NetB–Alpha toxin–TpeL' (NAT) construct. The construct was optimized for expression in *E. coli*. Selected regions of the NetB, alpha toxin and TpeL were fused together using two linkers (amino acids 173–194 and 309–330). A sequence of six Histidine was also added to the C-terminal of the construct for further protein purification.

of the sequence. All instability elements and the Cis-acting sites which may have affected the expression rate were removed.

Secondary structure prediction

A total of 455 residue chimeric protein was made of 126 helices, 120 strands and 204 random coils. Helices that located at the positions of 165–198 and 304–333 are corresponding to the two linkers' fragments. The secondary structure of the amino-acid sequences around the linkers was also highly similar to the native secondary structures of these sequences before fusing to the linker.

Tertiary structure prediction

The tertiary structure of four combinations consists of NetB, TpeL and alpha toxin were predicted by submitting their sequences to the I-TASSER server. Figure 2 represents the modeled sequential combination of NetB–Alpha toxin–TpeL (or NAT).

Data from all modeled combinations acquired. The combination NAT has the highest confidence score (*C*-score) (−1.20) comparing with the other three combinations. *C*-score is typically in the range of [−5,2] and higher values of *C*-score signify a model with a high confidence.

Prediction of mRNA structure

Potential folding of the mRNA for NAT construct was determined by mfold online software. The 5' terminus folding was similar to the other bacterial gene folding structure. No long stable hairpin pseudoknot is observed at the initializing 5′ nucleotides of the mRNA. The minimum free energy of the secondary structure formed by RNA was also predicted as −372.00 kcal/mol.

Prediction of B-cell epitopes

Toxins NetB, alpha toxin and TpeL were submitted to VaxiGen online software separately while the cutoff value of the software was set to 0.4 (Table II). Sequences with the values above this cutoff are predicted to be antigen by the software. The assigned score for NAT was 0.66, which is above the cutoff value. The epitopic regions were then mapped by BCEPRED online service for each individual toxin and the final designed chimeric protein.

Physico-chemical properties of the protein

The molecular weight of the chimeric protein was 52 kDa and the pI was 5.4. According to the Expasy's ProtParam, half-life of the protein was predicted to be >10 h. The chimeric protein was also predicted as stable.

Fig. 2 Prediction of the 3D structure of the chimeric protein based on comparative modeling. All three parts of the chimeric protein (i.e. NetB, alpha toxin and TpeL) are exposed to the environment. Two linkers are highlighted in white. The colors yellow, blue and red are corresponding to NetB, alpha toxin and TpeL, respectively.

Table II. Antigenic scores of different toxins

Toxin	Ag score
NetB	0.62
Alpha toxin	0.52
TpeL	0.35
NetB (amino acids 150–322)	0.74
Alpha toxin (amino acids 256–370)	0.72
TpeL (120 aa from C-ter)	0.34
NetB–Alpha toxin–TpeL	0.66

Protein expression and purification

After induction of transformed cells by IPTG, the chimeric protein was extracted and purified by nickel beads under denaturing condition according to the manufacturer instruction (QIAGEN Inc, USA, 2001). SDS-PAGE 12% revealed a single band between molecular weight markers of 45 and 66.2 kDa, which corresponds to the chimeric protein with the molecular weight of 52 kDa (Fig. 3).

For elimination of urea from the purified protein, the elution buffer containing the chimeric protein was dialyzed against dialysis buffer containing decreasing concentrations of urea from 8 M to zero, overnight. All concentrations of the urea buffers contained 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 10% Glycerol and pH 8.0.

CD analysis

The purified protein was subjected to CD analysis (Fig. 4) for determination of its secondary structure. The CD data were analyzed with the secondary structure estimation software as mentioned in Materials and Methods. The results of the percentage of secondary structure estimation are shown in Table III. Comparisons of the

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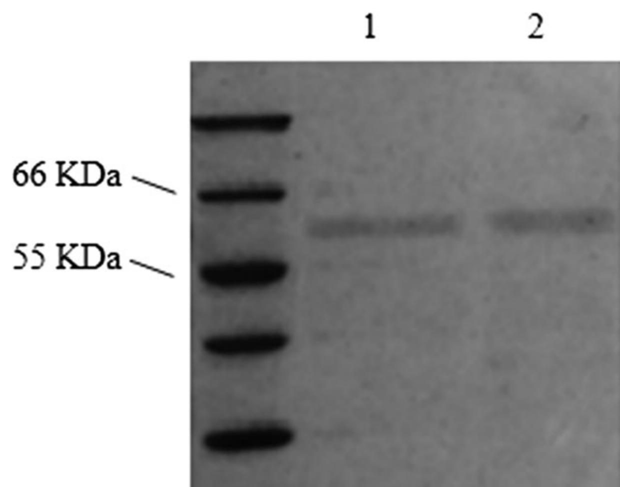

Fig. 3 Purification of the chimeric protein on SDS-PAGE. Lanes 1 and 2: purified chimeric protein in elutions one and two, respectively. The molecular weight of the chimeric protein is 56 kDa.

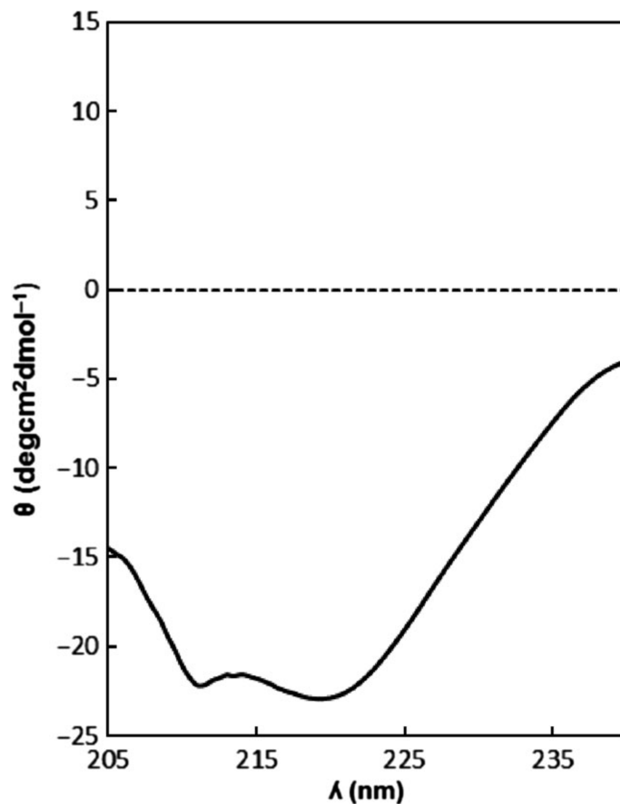

Fig. 4 The CD plot of the chimeric protein.

experimental data with that obtained by theoretical prediction are very similar and compatible.

Western blotting

Western blot analysis showed that the sera from the rabbit immunized with the chimeric protein can detect the whole chimeric protein as well as each of its subunits. As it is shown in Figure 5, all individual subunits are identified by the antibody, the chimeric protein is recognized more strongly compared with each of its separated subunits (lane 2).

Table III. Comparing the percentages of secondary structure of the chimeric protein predicted by bioinformatics tools with the data obtained by CD, using the secondary structure estimation software

Beta-turn	Random coil (%)	Beta-sheet (%)	Alpha-helix (%)	Secondary structure
Not calculated	45.33	26.67	28	Predicted
%15.9	41.4	25.7	17	CD

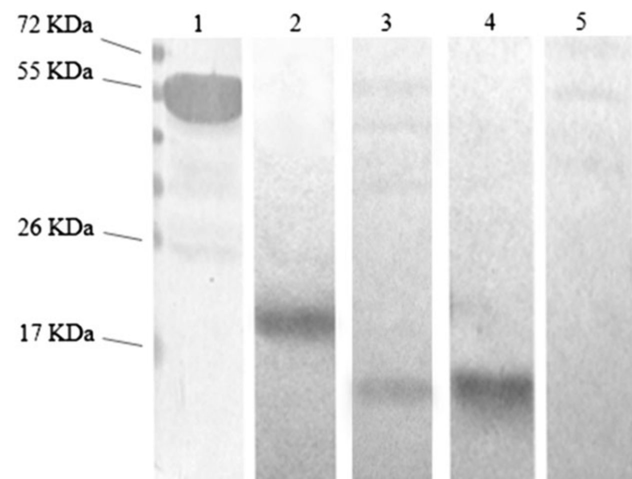

Fig. 5 Purification of the chimeric protein and its subunits by Ni-NTA and western blotting. Lane 1: western blotting of the purified chimeric protein (NetB–Alpha toxin–TpeL), lane 2: western blotting of the purified NetB, lane 3: western blotting of the purified alpha toxin, lane 4: western blotting of the purified TpeL, lane 5: uninduced total lysate of the *E. coli* bl21 (DE3). Western blotting was performed using rabbit primary antibody and conjugate anti-rabbit HRP.

Serum neutralizing test

Rabbits were injected twice with the purified chimeric protein: at Days 0 and 21. Two weeks after the second injection, serum samples were collected and analyzed for antibody presence. The potency experiment showed an increase in antibody levels against *C. perfringens* type A alpha toxin to 7 IU/ml, in immunized rabbits.

Other properties

In order to check the allergenicity of the chimeric protein, the sequence were submitted in AlgPred tool and SDAP (Ivanciuc *et al.*, 2003) allergen library. Neither AlgPred nor SDAP detected the sequence as a potential allergen (data are not shown).

Discussion

NE is characterized by chronic intestinal mucosal damage and lesions, reduced weight gain and enlarged liver as reviewed in Timbermont *et al.* (2011). However, increasing resistance to antibiotics highlights the importance of developing new strategies against bacterial infections. Among these strategies, recombinant vaccine technology is an effective approach for immunization against bacterial diseases like NE. Recombinant vaccines benefit from the feature

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of presenting more than one epitope, which could be especially crucial in multi-factorial diseases. An effective vaccine should have the ability to trigger immune response to bacterial antigens that are important in the microbial pathogenesis.

In this study, epitopes of three clostridial toxins including NetB, TpeL and alpha toxin were determined and linked together by two identical rigid linkers. The rigid linker induces minimal changes in the structure of the chimeric protein. These toxins are shown to be important in developing NE.

NetB is reported to be the most important toxin in developing NE. Due to the importance of the C-terminal amino acids in binding and cytotoxicity of the NetB (e.g. Arg 200 and Trp 262) (Savva *et al.*, 2013), 172 amino acids from C-terminal were selected for construction of the chimeric protein. Furthermore, recently it is reported that TpeL is playing an important role in severity of NE while its C-terminal truncated form is not cytotoxic (Coursodon *et al.*, 2012; Pauillac *et al.*, 2013). Thereby, 120 aa from C-terminal of the toxin were also selected. On the other hand, previous studies showed that vaccination against the recombinant full-length and C-terminal fragment of alpha toxin is effective against *C. perfringens* pathogenesis (Stevens *et al.*, 2004; Zeng *et al.*, 2011). Therefore, a sequence of 114 aa belongs to the C-terminal (amino acids number 256–370) was included in the chimeric protein construct. This strategy ensures that the raised antibody against the chimeric protein will recognize the same part in native NetB, TpeL and alpha toxin, which lead to prevention of their activity. The antigenicity of the selected regions was evaluated by VaxiJen online service. The obtained score for NetB and alpha toxin is higher than the native toxins, while TpeL has a lower score. However, the overall score for NAT is 0.66. This score is above each individual native toxin score and it is still evaluated as antigen (the software threshold was 0.4).

Different combinations of selected regions of these three toxins were modeled and tertiary structures of each combination were obtained. According to acquired data, the NAT combination regarded as the best folding form. The quality of the modeled structures in I-TASSER is evaluated by C-score. Higher values of this score signify a higher confidence. The modeled construct NAT has the higher C-score comparing with other three combinations. In this combination, all parts of the chimeric protein are predicted to be exposed to the environment. This regards as a desired feature for recombinant vaccine candidates since all parts of the suitable vaccine candidate should raise antibody. The template modeling score of the NAT is also above the threshold (>0.5), which verifies the correct topology of the modeled chimeric protein. Due to the high hydrophobicity of the TpeL, we preferred the construct not to be started with this toxin. So two combinations start with TpeL were intentionally discarded from these analysis.

The designed construct of NAT was expressed in *E. coli* as a host; however, the chimeric protein was insoluble and could not be purified under native condition. Although different modifications in growth (different temperatures) and induction conditions (including the different concentrations of IPTG and different ODs for induction) were examined (data are not shown), the chimeric protein was not solubilized. Since the chimeric protein was not expected to have any enzymatic activity, it was purified under denaturing condition and again refolded by dialysis approach mentioned in Materials and Methods section. However, the chimeric protein was highly expressed in *E. coli* such that its expression was resulted in inclusion body formation.

Secondary structure of the chimera was analyzed by CD. As it is shown in Table III, the data obtained by CD are very similar to the data obtained by prediction method, especially in the estimated amounts of beta-sheet and random coil. Accordingly, the chimera (chimeric protein) with <45% Alpha-helix and beta-sheet belongs to the family of proteins that are dominantly contain random coil structure.

As it is shown in Figure 5, the raised antibody is more sensitive to NetB and TpeL comparing with alpha toxin. These toxins are located at the two ends of the construct. According to the predicted tertiary structure, NetB and TpeL are more exposed to the environment than alpha toxin. So they may be better accessible by immune system. However, all three parts of the NAT are identified by the raised antibody.

On the other hand, different experiments confirmed that raising antibody against single NetB, alpha toxin or TpeL is not capable of yielding full protection against NE (Jang *et al.*, 2012; Keyburn *et al.*, 2013b). Recently, it is reported that administration of alpha toxin and NetB can protect broiler chickens against mild form of NE (Fernandes da Costa *et al.*, 2016). This highlights the importance of a chimeric vaccines that can neutralize more than one toxin at once. As it is shown, NAT can identify and bind to NetB, alpha toxin and TpeL separately. The ability of the antibody raised against NAT in neutralizing alpha toxin was also examined in a potency experiment. Our results showed an increased level of antibody against alpha toxin to ~7 IU/ml. Since the full length of toxins NetB and TpeL was not available for us, the potency experiments for these two toxins were not performed. However, western blot analyzing showed the ability of raised antibody in identifying NetB, alpha toxin and TpeL.

In conclusion, this is the first report describing the expression of the fusion NetB–Alpha toxin–TpeL in *E. coli* as a host. Based on our findings, this chimeric protein can be suggested as a multivalent vaccine candidate against NE caused by *C. perfringens*.

Supplementary data

Supplementary data are available at *PEDS* online.

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